



Modulation of interferon expression by hepatitis C virus NS5A protein and human homeodomain protein PTX1

Asish K. Ghosh,^{a,1} Mainak Majumder,^{a,1} Robert Steele,^a Ranjit Ray,^{b,c} and Ratna B. Ray^{a,b,*}

^a Department of Pathology, Saint Louis University, St. Louis, MO 63104, USA

^b Department of Internal Medicine, Saint Louis University, St. Louis, MO 63104, USA

^c Department of Molecular Microbiology and Immunology, Saint Louis University, St. Louis, MO 63104, USA

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Abstract

Hepatitis C virus (HCV) NS5A protein transcriptionally modulates a number of cellular genes. Since there is no evidence of binding of NS5A protein to DNA, it is likely to exert its activity in concert with cellular factor(s). In this study, we have identified a specific interaction of HCV NS5A with homeodomain protein PTX1 of human origin by a yeast two-hybrid interacting cloning system. The authenticity of this interaction was verified by mammalian two-hybrid assay, in vivo co-immunoprecipitation analysis, and from a colocalization study. Recently, murine PTX1 (mPTX1) has been shown to repress virus-induced murine interferonA4 promoter activity. Interferon- α alone or together with ribavirin is the only available therapy for HCV-infected patients. Therefore, we examined whether coexpression of NS5A and human PTX1 (hPTX1) proteins modulate human IFN- α promoter activity. An in vitro reporter assay by transfection of HepG2 cells with NS5A suggested an activation of IFN- α promoter to \sim 20-fold upon Newcastle disease virus (NDV) infection. Under similar experimental conditions, hPTX1-activated IFN- α promoter to \sim sevenfold, unlike mPTX1. However, cotransfection of NS5A and hPTX1 displayed a lower interferon promoter activity, probably for physical association between these two proteins. Subsequent study demonstrated that activation of IFN promoter by NS5A is associated with an increased expression of IRF-3. Further analysis revealed that ectopic expression of NS5A in HepG2 cells enhances endogenous IFN- α secretion and MxA expression upon induction with NDV. However, exogenous expression of hPTX1 did not significantly alter NS5A-mediated function in the stable transfectants. Taken together, these results suggested that the level of endogenous hPTX1 is not sufficient to block the function of NS5A for augmentation of virus-mediated IFN activity in HepG2 cells.

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Introduction

HCV is an important cause of morbidity and mortality worldwide, causing a spectrum of liver disease ranging from an asymptomatic carrier state to end-stage liver disease. The most important feature of HCV infection is the development of chronic hepatitis in a significant number of infected individuals and the potential for disease progression to hepatocellular carcinoma (Di Bisceglie, 1997; Hayashi et al., 1999; Jeffers, 2000; Saito et al., 1990), one of the commonest internal ma-

lignancies of mankind. HCV multiplication is sustained throughout the course of infection and during development of hepatocellular cancer (Kato et al., 1993; Noursbaum et al., 1995). Several HCV proteins have been implicated for virus-mediated pathogenesis (Reed and Rice, 2000). HCV genome contains a linear, positive-strand RNA molecule of \sim 9500 nucleotides (Choo et al., 1989; Kaito et al., 1994). RNA transcripts from cloned HCV cDNA can initiate infection leading to hepatitis by direct intrahepatic injection into chimpanzees (Kolykhalov et al., 1997; Yanagi et al., 1997). The HCV genome encodes a single polyprotein precursor of \sim 3000 amino acids (Choo et al., 1989), which is cleaved by both host and viral proteases (Grakoui et al., 1993; Hijikata et al., 1991) to generate at least 10 individual proteins.

The NS5 region of the polyprotein is composed of two major proteins, NS5A (p56) and NS5B (p65), which are gen-

* Corresponding author. Department of Pathology, Saint Louis University, 1402 South Grand Boulevard, 4th Floor, St. Louis, MO 63104. Fax: +1-314-771-3816.

E-mail address: rayrb@slu.edu (R.B. Ray).

¹ These authors contributed equally to this work.

erated as mature products by the action of the NS3 protease in conjunction with NS4A. NS5A exists as two phosphoproteins p56 and p58, which are phosphorylated at serine residues after the mature NS5A protein is released from the polyprotein (Kaneko et al., 1994; Tanji et al., 1995). Although phosphorylation of HCV NS5A occurs predominantly on serine, a low level of phosphorylation on threonine residues was also observed (Reed et al., 1997). Sequence comparison of the regions surrounding the sites of phosphorylation indicates an extremely high level of conservation between different strains of the virus but the biological significance of phosphorylation is still unclear. Results from replicon system supports that NS5A is an important component in HCV replication (Blight et al., 2000). NS5A protein has been implicated in HCV antiviral resistance (Tan and Katze, 2001). The sensitivity to IFN was correlated with mutations within the discrete region of NS5A and named IFN sensitivity determining region (ISDR) (Enomoto et al., 1995, 1996; Schiappa et al., 2002). However, the selective pressures evoked on HCV quasiespecies during IFN therapy appear to vary among different patients; ISDR locus per se does not function in a manner consistent with a major role in mediating IFN resistance (Polyak et al., 1998).

HCV NS5A protein is likely to play an important role in virus–host interaction. NS5A modulates a number of cell-cycle regulatory genes (Ghosh et al., 1999, 2000a; Majumder et al., 2001) and promotes murine fibroblasts to a transformed phenotype (Ghosh et al., 1999; Gale et al., 1999). Since NS5A does not bind to the DNA directly, it probably exerts its effect through other protein(s). NS5A downregulates the p21 gene by sequestering p53 in the cytoplasm (Majumder et al., 2001) and exerts antiapoptotic activity in mammalian cells (Gale et al., 1999; Ghosh et al., 2000b), as well as in transgenic mice (Majumder et al., 2002). Here, we demonstrated that HCV NS5A protein physically associates with homeodomain transcription factor PTX1. Since mouse PTX1 (mPTX1) represses virus-induced IFNA4 promoter (Lopez et al., 2000), we investigated the role of NS5A for modulation of IFN activity.

Results

Identification of NS5A interacting proteins by yeast two-hybrid screening

We undertook a search for host cellular proteins that interact with NS5A using yeast two-hybrid interaction cloning. HF7c yeast cells expressing NS5A were transformed with plasmid DNAs from a cDNA library. The candidate-interacting colonies were selected on the basis of their ability to grow in appropriate selection medium and turn on the *LacZ* reporter gene (Ghosh et al., 2000a). Thirty clones were grown in histidine-deficient selective medium and exhibited β -galactosidase activity. Plasmid DNA was isolated from 17 clones and re-transformed into HF7c yeast cells expressing Gal4-NS5A gene for positive interaction.

All clones exhibited growth in selective medium and β -galactosidase activity following retransformation. Upon further testing of these clones for interaction with a battery of heterologous baits in yeast, six clones were found to specifically interact with pGBT9-5A and not with other heterologous protein baits. These clones were sequenced and analyzed using the BLAST program. Sequence analysis revealed that two isolates represent an independent full-length cDNA clone with perfect homology to a human homeodomain protein PTX1 (GenBank Accession No. NM_002653).

Association of NS5A with human PTX1 in mammalian cells

The mammalian version of the conventional two-hybrid assay was used to ascertain whether NS5A associates with PTX1. For this purpose, we constructed mammalian expression plasmid vectors encoding a VP16-5A fusion protein and Gal4 DNA-binding domain protein fused to PTX1 (Gal4-PTX1). The mammalian two-hybrid assay was performed by transfecting HepG2 cells with a Gal4-responsive reporter gene (gE1bCAT) and pairwise combinations of the appropriate expression vectors. Reporter gene expression was determined by measuring CAT activity in cell lysates from each transfected culture. A significant increase in CAT activity was observed following coexpression of VP16-5A and Gal4-PTX1 hybrids (Fig. 1). However, CAT activity was not enhanced by coexpression of the VP16-5A and Gal4 vector or Gal4-PTX1 and VP16 Flag vector as a negative control (data not shown).

NS5A-hPTX1 form complex in vivo

A co-immunoprecipitation experiment was performed with lysates of HepG2 cells cotransfected with NS5A and CMVFlag-PTX1 to verify the ability of NS5A for association with PTX1 (Fig. 2). Cell lysates were incubated with pooled normal mouse sera as a negative control (Lane 1) or monoclonal antibody to Flag for immunoprecipitation (Lanes 2 and 3). The immunoprecipitates immobilized on protein A–Sepharose beads were separated by SDS–PAGE and blotted onto nitrocellulose membrane. Coprecipitation of NS5A with PTX1 was observed from the reactivity with specific antibody and the size of the NS5A protein. Cell lysates were also analyzed by Western blot and the presence of NS5A protein band was detected with specific antibody (Lanes 4 and 5). The protein bands with slower mobility (~85 kDa) detected in co-immunoprecipitated samples (Lanes 2 and 3) could not be definitely identified. The blot was stripped and, when reprobed with a monoclonal antibody to Flag, displayed the hPTX1 protein band (bottom). Therefore, specific association of NS5A and hPTX1 was observed using a mammalian two-hybrid assay and co-immunoprecipitation analysis.

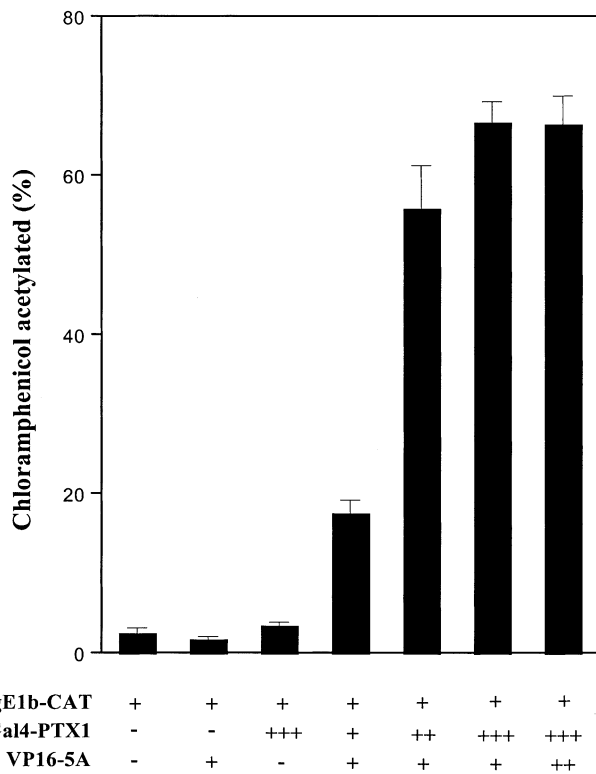


Fig. 1. Interaction of NS5A with PTX1 in mammalian two-hybrid system. HepG2 cells were transfected with 1 μ g gE1b-CAT reporter gene, 1 μ g of VP16-5A, and increasing doses of (1, 2, and 3 μ g, denoted by +, ++, and +++) of Gal4-PTX1. CAT assay was performed 48 h posttransfection. Amount of DNA was kept constant in each transfection by adding the empty vector DNA. PTX1-5A hybrid displayed a high level of CAT activity as compared to Gal4-PTX1 or NS5A alone. Chloramphenicol acetylated (1%) was arbitrarily assigned to the vector control.

NS5A colocalizes with hPTX1

We transfected cells with CMVFlag-PTX1 to examine its localization. Indirect immunofluorescence was performed using a monoclonal antibody to Flag and a predominant cytoplasmic localization of hPTX1 was observed (Fig. 3A). Next, cells were cotransfected with CMV-NS5A and CMVFlag-PTX1, and 48 h posttransfection cells were subjected to indirect immunofluorescence together with a mouse monoclonal antibody to Flag (Fig. 3B) and a rabbit antiserum to NS5A (Fig. 3C). Cytoplasmic localization of both hPTX1 and NS5A was observed. Confocal microscopy suggested colocalization of PTX1 and NS5A proteins predominantly in the cytoplasm of the cells (Fig. 3D), while control antibodies did not produce any detectable fluorescence (data not shown).

Mapping of hPTX1-binding domain in NS5A

To identify the region of NS5A responsible for binding with hPTX1, a mammalian two-hybrid assay was performed using Gal4-PTX1, VP16-5A, and its deletion mutants (Ma-

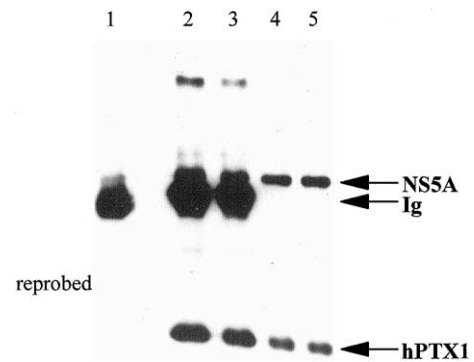


Fig. 2. Coimmunoprecipitation of hPTX1 with HCV NS5A. HepG2 cells were cotransfected with NS5A and CMVFlag-PTX1, and cells were lysed after 48 h of transfection. Cell lysates were immunoprecipitated with pooled normal mouse sera as a negative control (Lane 1) or with a monoclonal antibody to Flag in duplicate experiments (Lanes 2 and 3). Immunoprecipitates were separated by SDS–7.5% PAGE, blotted onto nitrocellulose, and probed with a rabbit antibody to NS5A. A secondary antibody conjugate (anti-rabbit IgG/horseradish peroxidase, Amersham) was used for detection of the peroxidase signal by chemiluminescence (ECL). The molecular weight of the NS5A band (58 kDa) was ascertained from the migration of standard protein molecular weight markers (Life Technologies). The positions of NS5A and Ig heavy chain are shown. Cell lysates were also analyzed by SDS–PAGE and the presence of NS5A protein band was detected using specific antibody (Lanes 4 and 5). The blot was reprobed with Flag monoclonal antibody for detection of hPTX1 (bottom).

jumder et al., 2001). A significant increase (~fivefold) in CAT activity was observed when full-length NS5A, VP16-5A₁₋₃₃₂, and VP16-5A₁₋₂₂₀ were cotransfected with Gal4-

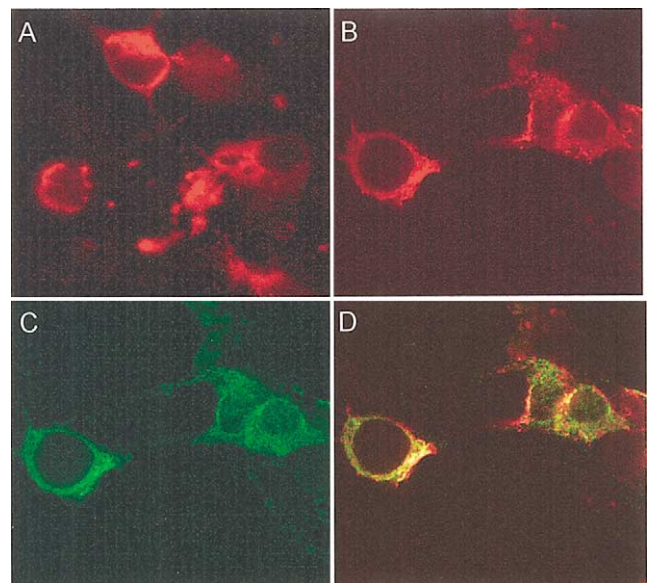


Fig. 3. Colocalization of hPTX1 and NS5A in HepG2 cells. Immunofluorescent staining using Flag epitope specific antibody exhibited cytoplasmic localization of hPTX1 in CMVFlag-PTX1-transfected HepG2 cells (A). Cells cotransfected with NS5A and PTX1 were stained with a monoclonal antibody to Flag for PTX1 (B) and a rabbit antibody to NS5A (C). Fluorescence images of B and C when superimposed digitally for fine comparison displayed colocalization of PTX1 and NS5A (D).

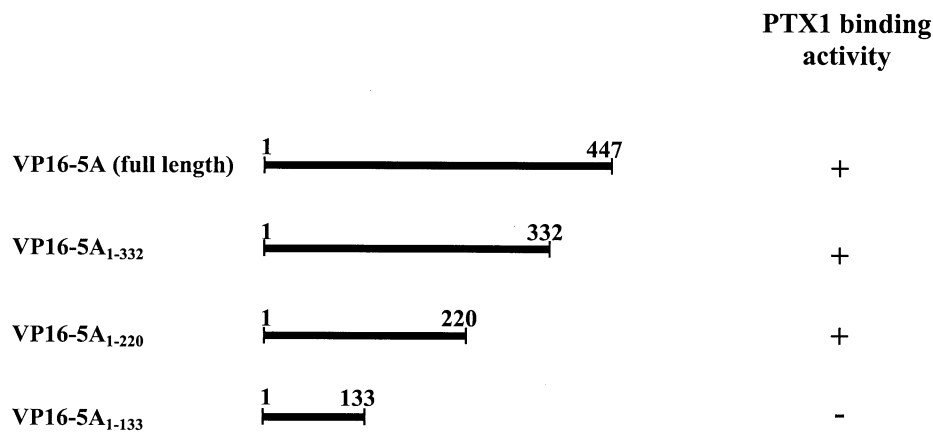


Fig. 4. Mapping of the hPTX1-binding domain in NS5A. Cells were cotransfected with gE1b-CAT reporter gene and pairwise combination of Gal4-PTX1 and various deletion mutants of NS5A in VPFlag vector. CAT assay was performed 48 h posttransfection. A significant increase of CAT activity (~fivefold) over basal level is indicated by +. Position of amino acids in the deletion mutants of NS5A is indicated by horizontal bars.

PTX1, but not with VP16-5A₁₋₁₃₃ (Fig. 4). However, CAT activity was not altered following coexpression of NS5A deletion mutants and Gal4 empty vector as a negative control. Results from this assay indicated that PTX1-interacting domain is localized between amino acid positions 133 and 220 of NS5A. These findings suggested association of PTX1 occurs through a specific region of NS5A, although the precise sequences responsible for this interaction remains to be identified.

NS5A and hPTX1 enhances human interferon promoter activity

We next investigated the role of hPTX1 and HCV NS5A on human IFN- α promoter. An in vitro reporter assay was performed by cotransfecting HepG2 cells with human IFNA4 promoter linked to luciferase reporter gene, empty vector, CMV-NS5A, and/or CMVFlag-PTX1. Cells were infected with NDV (m.o.i. 5) after 16 h of transfection or left uninfected and luciferase activity was measured 24 h postinfection. Results suggested that expression of NS5A or hPTX1 activates virus-induced human IFNA4 promoter in HepG2 cells, while significant change was not observed in uninfected cells (Fig. 5). However, coexpression of NS5A and hPTX1 reduced human IFNA4 promoter activity as compared to NS5A or PTX1 alone, suggesting an association of these two proteins may prevent promoter activation. Similar results were also obtained when cells were transfected with IFNA14 promoter (data not shown). Results from these experiments suggested that the presence of NS5A or hPTX1 activates IFNA4 promoter upon induction with NDV.

NS5A enhances IRF-3 expression

The IRF-3 and IRF-7 have been identified as direct transducers of virus-mediated signaling and play a critical

role in the induction of Type I IFN genes (Sen, 2001). IRF-3 is expressed widely in cells and demonstrated a unique response to virus infection. Unlike IRF-3, IRF-7 is not expressed constitutively in cells and induced by IFN or virus infection. To further understand how NS5A regulates the IFNA4 promoter activity, we examined the expression level of IRF-3 and IRF-7. HepG2-neo or HepG2-NS5A cells were transiently transfected with hPTX1. Cells were infected with NDV (m.o.i. 5) and cell lysates were collected 24 h postinfection. Western blot analysis was performed using specific antibody to IRF-3 (Fig. 6). Our results sug-

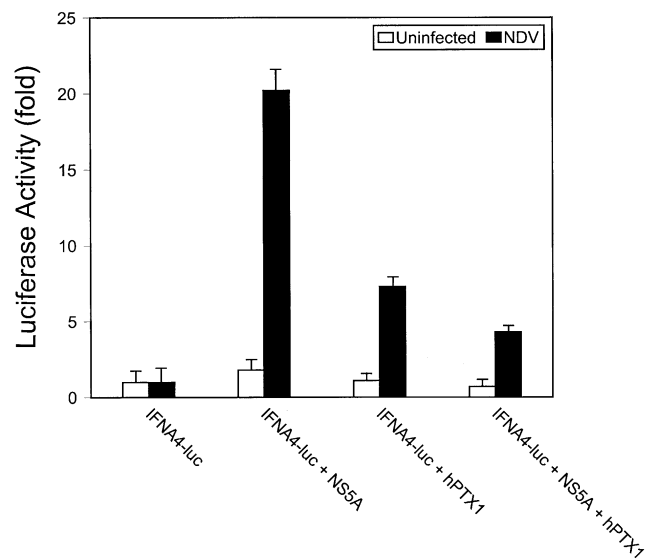


Fig. 5. Activation of virus-induced human IFNA4 promoter by NS5A and hPTX1. Cells were cotransfected with human IFNA4-luc reporter gene and the indicated expression constructs. The total amount of DNA was kept constant by addition of empty vector in each transfection. Cells were infected with NDV as described under Materials and Methods and cell extracts were prepared for luciferase assay. In each set of experiments, triplicate transfections were performed and basal value was arbitrarily set at 1.

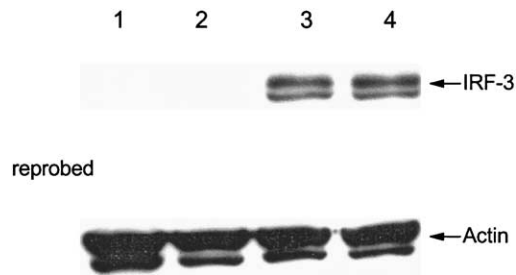


Fig. 6. HCV NS5A protein enhances IRF-3 expression in HepG2 cells upon NDV induction. HepG2-neo control cells (Lane 1), transfected with hPTX1 (Lane 2), and HepG2-NS5A cells (Lane 3) or HepG2-NS5A cells transfected with hPTX1 (Lane 4), were induced with NDV for 24 h. Cell lysates were analyzed by Western blot using a polyclonal antibody to IRF-3 (Santa Cruz). The molecular weight of IRF-3 band was ascertained as ~50 kDa from the migration of standard protein molecular weight markers (Life Technologies). The blot was reprobed with antibody to actin for comparison of protein level.

gested a significant increase of IRF-3 expression (~20-fold) in HepG2-NS5A cells (Lane 3) as compared to control HepG2-neo cells. HepG2-NS5A cells transiently transfected with hPTX1 also displayed an increased level of IRF-3 expression (Lane 4). Unlike the results of IFNA4 promoter regulation in an *in vitro* transient transfection assay, the lack of IRF-3 inhibition by coexpression of NS5A-hPTX1 may arise from the difference between the expression level of NS5A and hPTX1 in HepG2-NS5A stable transfectants. On the other hand, HepG2-neo cells alone (Lane 1) or when transfected with hPTX1 (Lane 2) did not display a detectable IRF-3 expression upon NDV infection. Therefore, the mechanism of virus-induced hPTX1-mediated IFNA4 promoter activation may follow a different mechanism and remains to be elucidated. We also examined the level of IRF-7 under similar experimental conditions and did not observe a detectable expression. This could be due to no or very little expression of IRF-7 in human liver cells (Au et al., 1998) and may explain the relative weakness of hepatocytes for IFN stimulation (Keskinen et al., 1999).

NDV-mediated IFN- α secretion is enhanced by NS5A

To determine the role of NS5A in the induction of NDV-mediated IFN expression, HepG2 cell-culture medium was examined for secretion of IFN- α by ELISA (R&D Systems, MN). HepG2-neo or HepG2-NS5A/hPTX1 cell-culture supernatant was collected 48 h postinfection with NDV. A detectable amount of human IFN- α (sensitivity 12.5 pg/ml) in vector-transfected HepG2 cell supernatant was not observed (Table 1). This may be due to intrinsic property of hepatoma cells with poor ability to produce and respond to type I IFNs, which may contribute to their inability to efficiently resist viral infections (Keskinen et al., 1999). Interestingly, cells transfected with hPTX1, although displayed ~sevenfold increase in IFNA4 promoter activity,

Table 1

NDV-Mediated Induction of IFN- α in HepG2 Cells Expressing HCV NS5A

Cells	IFN- α production (pg/ml) ^a	
	–NDV	+NDV
HepG2-neo	<12.5	<12.5
HepG2-neo + hPTX1	<12.5	<12.5
HepG2-NS5A	<12.5	208 \pm 7
HepG2-NS5A + hPTX1	<12.5	165 \pm 6

^a The value < 12.5 pg/ml denotes the lower limit of detection of IFN- α level. Results are presented from mean of three independent experiments.

did not induce a detectable amount of IFN- α secretion in the culture medium. This could be due to higher sensitivity of the luciferase activity in comparison to ELISA-based IFN assay. On the other hand, ectopic expression of NS5A in HepG2 cells significantly enhanced NDV-mediated stimulation of IFN- α (208 \pm 7 pg/ml) and corroborated with a significant increase in IFNA4 promoter activation (~20-fold). However, exogenous expression of hPTX1 in HepG2-NS5A cells slightly decreased virus-mediated IFN- α secretion (165 \pm 6 pg/ml). Together, these results suggested that the level of endogenous hPTX1 is not sufficient to block the function of NS5A for augmentation of virus-induced IFN activity in HepG2 cells.

IFN- α is produced by most cells in response to viral infection. The binding of IFN- α to its receptor mediates a signal cascade that results in the transcriptional induction of IFN-induced genes. Although most of these genes are of unknown function, several have demonstrable antiviral activity. MxA gene is activated as a secondary response to NDV-induced interferon (Bazzigher et al., 1992). MxA is a guanosine triphosphatase and inhibits the replication of RNA viruses. Since we could not detect IFN- α secretion in NDV-induced HepG2 cell-culture medium, the cell-associated downstream molecule MxA of IFN pathway was examined. Upon NDV induction, an almost undetectable level of MxA was observed in HepG2-neo control cells by Western blot analysis using a specific antibody (Fig. 7). On the other hand, HepG2-NS5A cells displayed a significant level of MxA expression upon NDV induction and exogenous expression of hPTX1 did not alter MxA expression in HepG2-NS5A cells. Protein concentration was normalized by reprobing the blot with an antibody to actin. Basal-level

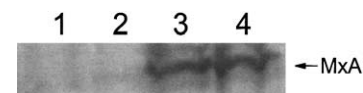


Fig. 7. HCV NS5A protein enhances MxA expression in HepG2 cells upon NDV induction. HepG2-neo control cells (Lane 1) transfected with hPTX1 (Lane 2), and HepG2-NS5A cells (Lane 3) transfected with hPTX1 (Lane 4), were induced with NDV for 24 h. Cell lysates were analyzed by Western blot using monoclonal antibody to MxA. The molecular weight of MxA band was ascertained as ~76 kDa from the migration of standard protein molecular weight markers (Life Technologies).

MxA was not detected in uninfected HepG2-neo or HepG2-NS5A cells (not shown in figure). This result corroborates with our findings (Table 1) that endogenous IFN- α production is enhanced in HepG2 cells expressing NS5A.

Discussion

Our observations suggest that HCV NS5A protein from genotype 1a physically associates with homeodomain protein PTX1 and colocalizes in the cytoplasm of HepG2 cells. Functional data indicate that NS5A or hPTX1 alone enhances NDV-induced IFNA4 promoter activity in an *in vitro* transient transfection assay. However, coexpression of NS5A and hPTX1 proteins reduces IFNA4 promoter activity close to basal level probably for physical association between these two interacting proteins. PTX1 gene is expressed during embryogenesis, in adult tissues, and in different cell lines (Lopez et al., 2000; Tremblay et al., 2000). Homeodomain transcription factors function by positively or negatively regulating spatial and temporal patterns of gene expression. The activity of PTX1 as a positive transcriptional regulator is synergized by cell-restricted transcription factors (Poulin et al., 1997; Szeto et al., 1996; Tremblay et al., 1999; Tremblay and Drouin, 1999). Oct-1, a homeodomain transcription factor, activates different promoters and is also involved in repression of the hPit1 gene (Delhase et al., 1996). Human homolog of mPTX1 represses virus-induced murine IFNA4 promoter activity by binding to promoter sequences (Lopez et al., 2000). Sequence comparison suggested that human and mouse IFNA4 promoters are different. Furthermore, the sequences (DNRE) where mPTX1 binds to the murine IFNA4 promoter are not present in human IFNA4 promoter. Therefore, these two promoters may be regulated by distinct mechanisms. It is possible that hPTX1 modulates human IFNA4 promoter by DNA-independent protein–protein interaction through other cellular factor(s). Our results suggest that HCV NS5A enhances IRF-3 expression upon NDV infection; however, IRF-7 expression could not be observed. At present, we do not know whether elevation of IRF-3 by NS5A upon NDV induction is due to the altered mRNA induction or protein stability. It is possible that NS5A may disrupt a cellular proteasome pathway to enhance IRF-3 stability (Servant et al., 2001). Thus, the mechanism of IRF-3 elevation by NS5A remains to be investigated. Both IRF-3 and IRF-7 are regulated in part by virus induction; however, these transcription factors impart complementary rather than redundant functional roles in cytokine gene activation (Lin et al., 2000a). In fact, human IRF-7 is expressed effectively only in lymphoid tissues, peripheral blood mononuclear cells, and some cell lines of lymphoid origin, and its transcription can be further stimulated by treatment with IFN (Au et al., 1998). However, none or very little expression of IRF-7 could be detected in established cell lines of fibroblast or epithelial origin including liver. This could account for

undetectable IRF-7 expression in NDV-infected HepG2 transfectants.

At present, the only approved therapy for chronic HCV infection is IFN- β with or without ribavirin (Hoofnagle and Di Bisceglie, 1997; McHutchison et al., 1998; Moradpour and Blum, 1999). The sustained antiviral response rate of IFN- β monotherapy is low (~30%) and combination therapy with ribavirin is higher (~50%) in treated patients (Poynard et al., 1996; Alter and Seeff, 2000; Jaeckel et al., 2001; Rosen et al., 2002). The functional role of HCV proteins in the regulation of interferon signaling pathways is important to understand. HCV NS5A has been implicated for interference of IFN-mediated antiviral activity (Tan and Katze, 2001). However, a hepatoma cell line (Huh7) adapted HCV replicons from genotype 1b using IRES from EMCV displayed high susceptibility to IFN- α treatment, independent of a number of mutations in NS5A (Bartenschlager, 2002; Blight et al., 2000; Guo et al., 2001). Inhibition of the virus-replicative cycles from HCV 1a and 1b by IFN- α in hepatocytes has also been suggested (Chung et al., 2001; Castet et al., 2002). Thus, the role of mutations in NS5A protein during clinical response to IFN- α therapy and the *in vitro* effects of NS5A on the IFN system are not yet well understood. Recently, IRES from EMCV has been suggested to be sensitive to IFN, while HCV IRES is resistant (Koev et al., 2002). This might prevent the early defense response which may allow the completion of the first stages of virus life cycle, as a result virus could replicate in cells. Subsequently, viral proteins in concert with host proteins may trigger the signal transduction pathway in determining resistance or sensitivity to IFN.

Although we have examined the virus-induced activation of IFNA4 promoter among 14 subtypes of IFN- α promoters, HepG2 cells expressing NS5A displayed a significant level of endogenous IFN- α production and MxA expression as a secondary response to IFN in cells. In contrast, endogenous IFN- α secretion or induction of MxA could not be detected after NDV infection of vector-transfected HepG2 cells. This result is in agreement with Keskinen et al., (1999), where they also observed that HepG2 cells infected with influenza A or Sendai virus did not produce a detectable level of IFN- α . We do not have conclusive evidence as to whether HCV stimulates endogenous IFN- α production for activation of innate antiviral host responses (Castelruiz et al., 1999; Jakschies et al., 1994). However, expression of several IFN-induced genes, MxA, IP-10, IFI-56K, and p44, in the liver of HCV-infected patients was found to be significantly enhanced (Patzwahl et al., 2001). IFN-induced genes are also stimulated during HCV RNA replication within the liver of acutely infected chimpanzees (Bigger et al., 2001). These results indicate that HCV may be an activator of IFN-inducible genes. While our manuscript was in preparation, Fredericksen et al., (2002) reported that continuous expression of nonstructural genes from HCV 1b replicon enhances IFN- α production upon Sendai virus infection in Huh7 cells. However, the contribution of specific

nonstructural proteins were not examined. To our knowledge, this is the first report on activation of human IFN α 4 promoter and IFN- α production upon virus induction in hepatocytes expressing NS5A. IFNs are important regulators of genes encoding for proteins involved in the signal transduction of IFN and other cytokines. IFN- α/β is produced by virus-infected host cells and constitutes the primary response against infection. Both IFN- α and IFN- α/β follow similar downstream pathways for antiviral activity. HCV may induce interferon production upon infection of target cells. However, the downstream pathways for IFN-mediated anti-HCV activity in human hepatocytes remains to be elucidated. In fact, ~20% of the HCV-infected patients naturally clear virus infection, although the mechanism of this virus clearance is not well understood. Enhancement of IFN- α along with other immunological mechanisms may play a significant role in virus clearance in these individuals. However, our observations should not be interpreted to mean that activation of IFN- α production by HCV NS5A is the only mechanism for viral clearance or sensitivity to IFN- α therapy. A recent finding suggests that IFN- α can block HCV replication in primary human hepatocyte culture (Castet et al., 2002). These observations and clinical outcome of IFN- α trial (Manns et al., 2001) underscore critical investigations in elucidating the mechanism of both responsiveness and resistance of HCV and the altered nature of immune responses during IFN- α treatment.

Materials and Methods

Yeast two-hybrid screening

In search of an interacting host protein with HCV NS5A, a yeast two-hybrid screening was carried out in which the entire cDNA-coding region of HCV NS5A (genotype 1a, H strain) was fused in-frame with the Gal4 DNA-binding domain into the pGBT9 plasmid vector (Clontech) as a bait for screening a cDNA library (Ghosh et al., 2000a). Autonomous activation of the reporter gene was not observed by NS5A. The screening procedure was previously described (Ghosh et al., 2000a). At least 2.5×10^6 colonies were screened and colonies were selected on agar plates lacking histidine, tryptophan, and leucine over a 7-day period. Positive yeast transformants were picked up and replated for β -galactosidase assay by colony-lift filter procedure. A positive interaction was determined by the appearance of blue colonies. The β -gal positive colonies were grown on a selective medium for plasmid DNA isolation. Isolated plasmids were transformed into *Escherichia coli* KC8 strain and selected for the activation domain plasmids on M9-leu⁻ agar plates. The potential NS5A interacting cDNA inserts were retransformed into HF7c yeast strain bearing pGBT9-5A fusion gene and were grown on an appropriate selective medium for β -gal assay. Positive interacting cDNA clones were analyzed by nucleotide sequencing us-

ing an automated sequencer (ABI). Nucleotide and predicted amino acid sequences were compared with known protein sequences deposited in GenBank by BLAST analysis.

Mammalian two-hybrid analysis

Full-length PTX1 was cloned in-frame with the Gal4 DNA-binding domain under the control of SV40 promoter (Gal4-PTX1). A hybrid polypeptide containing the transactivation domain of herpesvirus VP16 fused to NS5A was also constructed (VP16-5A) using the mammalian expression vector VPIflag7 (Ghosh et al., 2000a). HepG2 cells (with no evidence of hepatitis B virus genome, procured from ATCC) were cotransfected with 1.0 μ g Gal4 responsive reporter gene (gE1b-CAT), 1.0 μ g VP16-5A, and varying concentrations of Gal4-PTX1 effector plasmids. CAT assay was performed 48 h posttransfection as previously described (Ghosh et al., 2000a). In all the transfection experiments, β -galactosidase gene was included to normalize the transfection efficiency.

Co-immunoprecipitation

HepG2 cells grown in 35-mm plates were transfected with 1 μ g each of the CMV-NS5A and CMVFlag-PTX1 (human PTX1 cDNA with N-terminal Flag epitope cloned under the control of CMV promoter) using lipofectamine transfection system (Life Technologies, Inc). Cell lysates were prepared 48 h posttransfection in 0.3 ml of low-stringency lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.6, 0.1% Nonidet P-40, 5 mM EDTA) containing protease inhibitors (2 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 mM phenylmethylsulfonylfluoride). Each cell lysate was incubated with Flag monoclonal antibody and immobilized onto Protein A-Sepharose CL-4B beads (Pharmacia). Immunoprecipitates were subjected to SDS-PAGE, followed by Western blot analysis using either NS5A or Flag-specific antibody.

Immunofluorescence study

HepG2 cells grown on glass coverslips were transfected with NS5A and/or CMVFlag-PTX1 using lipofectamine. Cells were washed 48 h posttransfection and fixed with 3.7% formaldehyde in PBS for 30 min. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed, and incubated with either anti-NS5A rabbit polyclonal or anti-Flag mouse monoclonal antibody for 1 h at room temperature. Cells were washed and incubated with fluorochrome-conjugated secondary antibodies for 30 min at room temperature. Finally, cells were washed and mounted for confocal microscopy (Bio-Rad 1024). Fluorescent images were superimposed digitally to allow fine comparison. Colocalization of green (FITC) and red (TRITC) signals in a single pixel produces a yellow color, while separated signals

remain green or red. Cells did not display a detectable staining when negative control sera were used.

In vitro reporter gene assay

HepG2 cells were cotransfected with the human IFNA4-luc reporter gene (Lin et al., 2000b) and/or CMV NS5A with or without CMVFlag-PTX1 plasmid DNA using lipofectamine. After 16 h of transfection, cells were either infected with NDV (m.o.i. 5) or left uninfected. Luciferase assay was performed 20–24 h postinfection as previously described (Ghosh et al., 2000a).

Western blot for MxA

MxA was analyzed by Western blot as described earlier (Schuster et al., 1996). Briefly, transfected HepG2 cells were induced with NDV for 24 h prior to lysis by SDS–PAGE buffer. Cell lysates were resolved on a SDS–8% polyacrylamide gel, transferred to nitrocellulose membrane, probed with a mouse monoclonal antibody to MxA, and detected by chemiluminescence (ECL, Amersham).

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